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The Relative Role of Bacterial Cell Wall and Capsule in the Induction of Inflammation in Pneumococcal Meningitis

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The relative contribution of bacterial components to the induction of inflammation during *Streptococcus pneumoniae* meningitis is unknown. Several strains of pneumococci with differences in cell surface characteristics (capsule or cell wall) were compared for the effect on the inflammatory response evoked during infection of the cerebrospinal fluid (CSF) in vivo. The presence of bacterial capsular polysaccharide was not necessary for bacterial growth in CSF in vivo but correlated with greater CSF bacterial density. CSF inflammatory changes began to appear when the bacterial concentration exceeded 10^5 cfu/ml, regardless of the pneumococcal strain. CSF inflammatory changes could be invoked by cisternal instillation of 10^5–10^6 cell equivalents of whole, heat-killed unencapsulated strains or their isolated cell walls but not by similar concentrations of heat-killed encapsulated strains or isolated capsular polysaccharide. Hypoglycorrhachia was observed only during inflammation caused by live bacteria. The inflammatory response characteristic of naturally acquired pneumococcal meningitis can be reproduced by challenge with both encapsulated and unencapsulated bacteria. The bacterial cell wall appears to be the most potent pneumococcal surface component in inducing CSF inflammation.

Many steps in the pathogenesis of pneumococcal meningitis remain unclear. This is particularly true in regard to the contribution of bacterial components to the induction of the host inflammatory response once bacteria gain access to the cerebrospinal fluid (CSF). An understanding of which bacterial component(s) incites the host response is important since products of inflammation that are released during infection may injure host tissues and be detrimental to the recovery from disease [1–3]. This information may provide important clues as to why mortality from pneumococcal disease remains at 20% despite the development of new, highly bactericidal antibiotics [4]. We present evidence that of the major surface components, the cell wall, and not capsular polysaccharide, appears to be a powerful inducer of the meningeal inflammatory response.

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Materials and Methods

Bacterial strains. *Streptococcus pneumoniae* strain A_{II} is an encapsulated Rockefeller University laboratory strain (Type II). Strains R_6 and lyt 4–4 (an autolysin-deficient tolerant strain) are unencapsulated and derived from strain A_{II} [5]. Strain S_{III} is a clinical isolate (Type III) obtained from Dr. M. Sande (San Francisco General Hospital Medical Center, San Francisco, Calif). Strain 8249 (Type XIX) is a penicillin-tolerant and penicillin-resistant clinical isolate obtained from Dr. Koornhof (Johannesburg, South Africa).

Preparation of inocula. Bacteria were grown for 6–8 hr in a synthetic medium [6] supplemented with 0.1% yeast extract (Difco, Detroit, Mich) to midlogarithmic phase. The cells were centrifuged at 2,000 *g* for 10 min at 4 C, then washed, resuspended, and diluted in pyrogen-free saline (CIBA-GEIGY, Basel, Switzerland), and injected into the cisterna magna. Inoculum was titrated for cfu on trypticase soy broth (TSB) agar plates containing 5% sheep blood.

Meningitis model. Male chinchilla rabbits weighing 2 kg (Thom Farm, Biebarach an der Riss, Federal Republic of Germany) were prepared according to the method of Dacey and Sande [7]. Unless otherwise noted, four rabbits were tested for each varia-

ble examined. Under anesthesia with 30 mg/kg iv pentobarbital (Abbot AG, Zug, Switzerland), a dental acrylic helmet was attached to the crown of the calvarium. A minimum of 24 hr later, the rabbits were again anesthetized with 1.75 g/kg sc ethyl carbamate (Urethan®; Fluka, Buchs, Switzerland) and 15 mg/kg iv pentobarbital and positioned in a sterotaxic frame. A spinal needle measuring 25 gauge by 3.5 inch (Becton-Dickinson, St. Augustine de Quadalix, Spain) was introduced into the cisterna magna and fixed in position on the frame; 0.3 ml of CSF was withdrawn and 0.2 ml of bacteria or bacterial component in pyrogen-free diluent was introduced. Serial 0.3-ml samples of CSF were obtained over the subsequent 12-hr of anesthesia. For sampling beyond this time, the rabbits were reanesthetized with pentobarbital (as above) as required.

Immediately upon withdrawal, CSF samples were analyzed for number of cfu as above. Red and white blood cell counts were determined in a Sysmex Microcell Counter (CC-108; TDA, Kobe, Japan). When red blood cells were detected, the number of leukocytes was adjusted to correct for contamination of CSF by blood. The remainder of each CSF sample was centrifuged (10,000 *g*, 3 min) with an Eppendorff microcentrifuge, and the supernatant was frozen and stored at −70 C for subsequent chemical analysis. Glucose was determined by a microadaptation of a commercial assay kit (Test-combination glucose; Boehringer Mannheim, GmbH, Federal Republic of Germany), protein by the Lowry method [8], and lactic acid by a microadaptation of a commercial assay kit (Sigma UV test 826-UV; Sigma Chemical, St. Louis, Mo.).

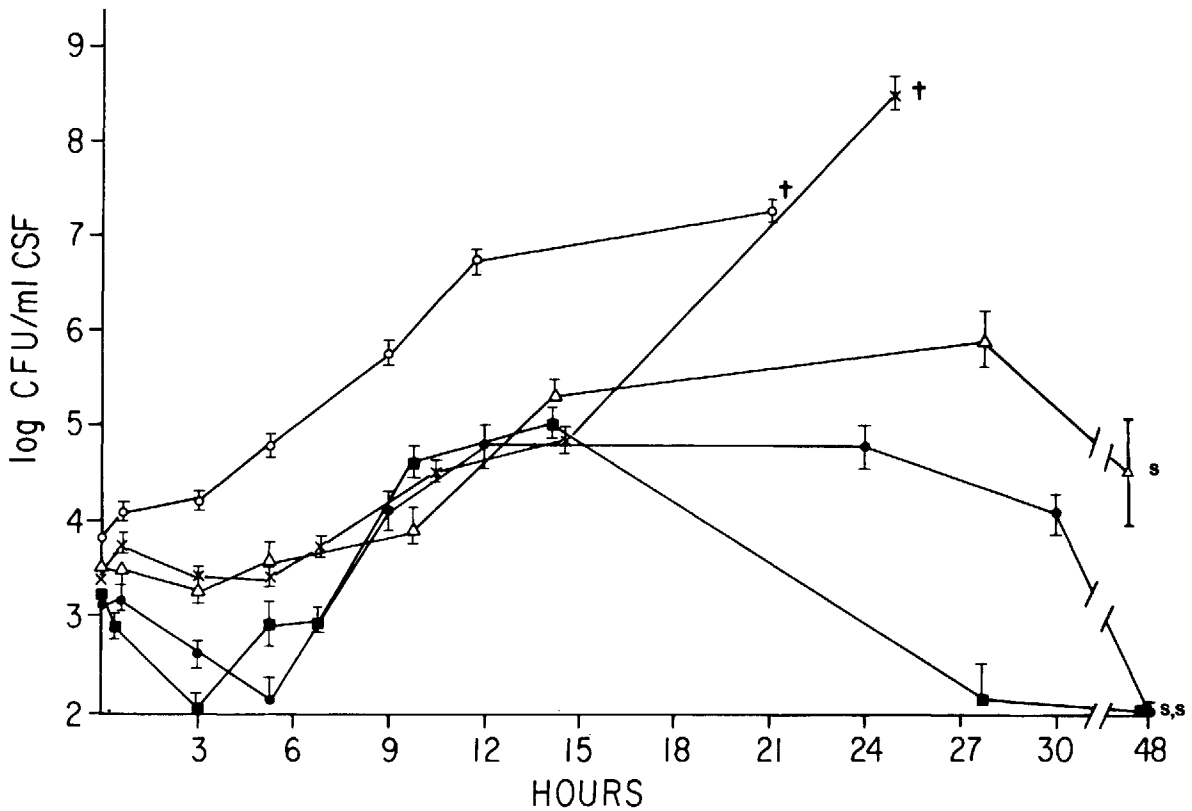

Figure 1. Growth rate of five strains of *S. pneumoniae* inoculated intracisternally into rabbits. Bacteria were prepared as outlined in Materials and Methods, and the inoculum was adjusted to ~ 10^4 cfu/ml. For each strain, 0.2 ml of this suspension was introduced into the cisterna magna of each of four rabbits. The numbers of cfu/ml in CSF were then observed over time. Strain R_6 (●); strain 8249 (x); strain S_{III} (○); strain lyt 4-4 (■); and strain A_{II} (△). Each data point is the mean ± standard error of the mean (SEM) of four rabbits. † = all animals in the group died; and s = all animals in the group survived. LD_{50} for each strain is as follows: R_6 = 10^7; A_{II} = 10^6; S_{III} = 10^2; and 8249 = 10^2.

Preparation of killed bacteria and bacterial components. *S. pneumoniae* strain A_{II}, S_{III}, or R_6 was grown until the bacterial concentration reached 10^8 cfu/ml (verified by cfu titration). A 10-ml culture was placed in a boiling water bath for 10 min and then centrifuged at 2,000 *g* for 10 min, washed and resuspended in pyrogen-free saline. The recovery of bacterial cells (i.e., cell-sized particles) was determined by a Coulter Counter Model T AII (Coulter Electronics, Harpenden, Hertfordshire, England).

Pneumococcal capsular polysaccharide was prepared by dissolving the 14-component pneumococcal polysaccharide vaccine (Pneumovax®; Merck, Sharp & Dohme, Rahway, NJ) in pyrogen-free saline, and the solution (5 mg of polysaccharide/ml) was dialyzed extensively against pyrogen-free saline in the cold. (Dialysis membrane was prepared by boiling, soaking in 0.5 *M* EDTA, and then rinsing in pyrogen-free saline.) Purified pneumococcal cell walls were prepared as described previously [5]. The cell wall preparations were weighed and resuspended in pyrogen-free saline at a concentration of 1 mg/ml. The suspensions were homogenized by a brief (2–3 sec) exposure to sonication (Branson Sonifier, model W 225 R; Heat Systems Ultrasonics, Farmingdale, NY) before inoculation into rabbits.

Statistics. Statistical differences between the mean values for two groups of rabbits were calculated by using the two-tailed *t* test.

Results

Growth of encapsulated and unencapsulated pneumococci in vivo. All pneumonococcal strains tested had the capacity to grow in CSF (figure 1). Encapsulated strains did not have shorter generation times. The lag time before the onset of growth and the growth rates were characteristic of each strain and did not correlate with the presence of bacterial capsule. Lethal meningitis could be induced despite lack of capsular polysaccharide when inocula of high bacterial titer (10^7 cfu/ml) were used. The presence of capsule was associated with higher and more sustained bacterial density in CSF.

Variation in CSF chemistry in response to encapsulated and unencapsulated pneumococci. The time course and magnitude of the cellular and chemical changes in CSF following inoculation with two encapsulated strains (Types III and XIX), unencapsulated (R_6) pneumococci, and pyrogen-free saline are shown in figure 2. These data were obtained in parallel with the growth curves of figure 1 and can be compared directly. Saline alone did not induce abnormal changes in CSF cytochemistry. Leukocytes

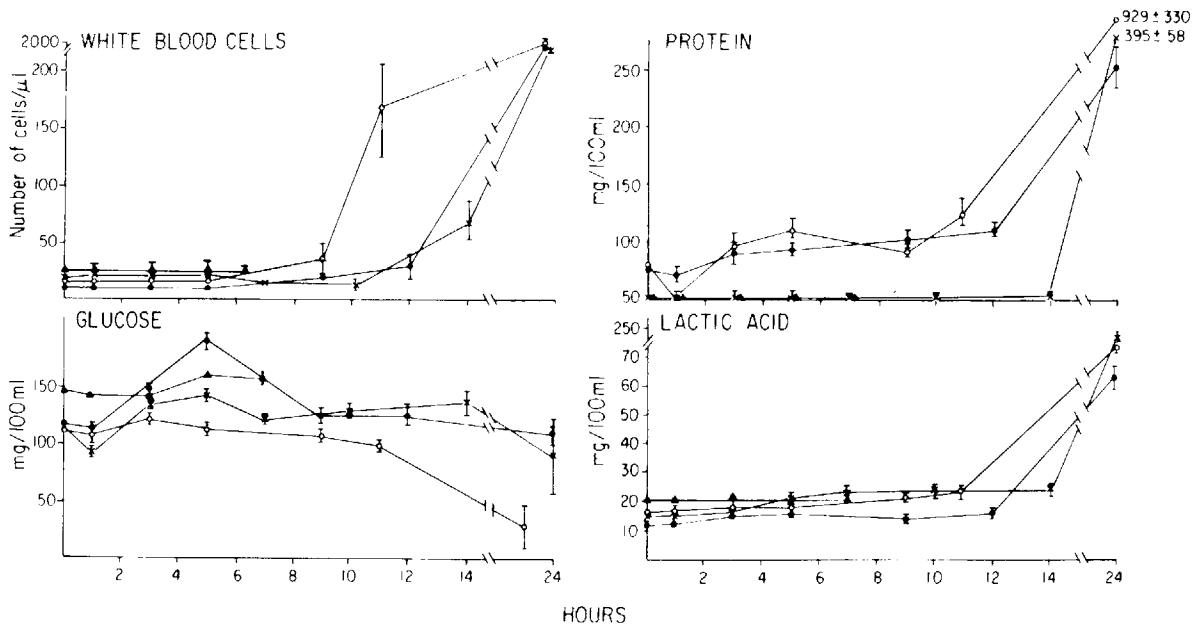

Figure 2. CSF cytochemical profile following intracisternal inoculation of three strains of *S. pneumoniae* into rabbits. Samples were collected in parallel with those described in figure 1. Each data point represents the mean (± SEM) of samples from four rabbits. Strain R_6 (●); strain 8249 (x); strain S_{III} (○); and saline (▲).

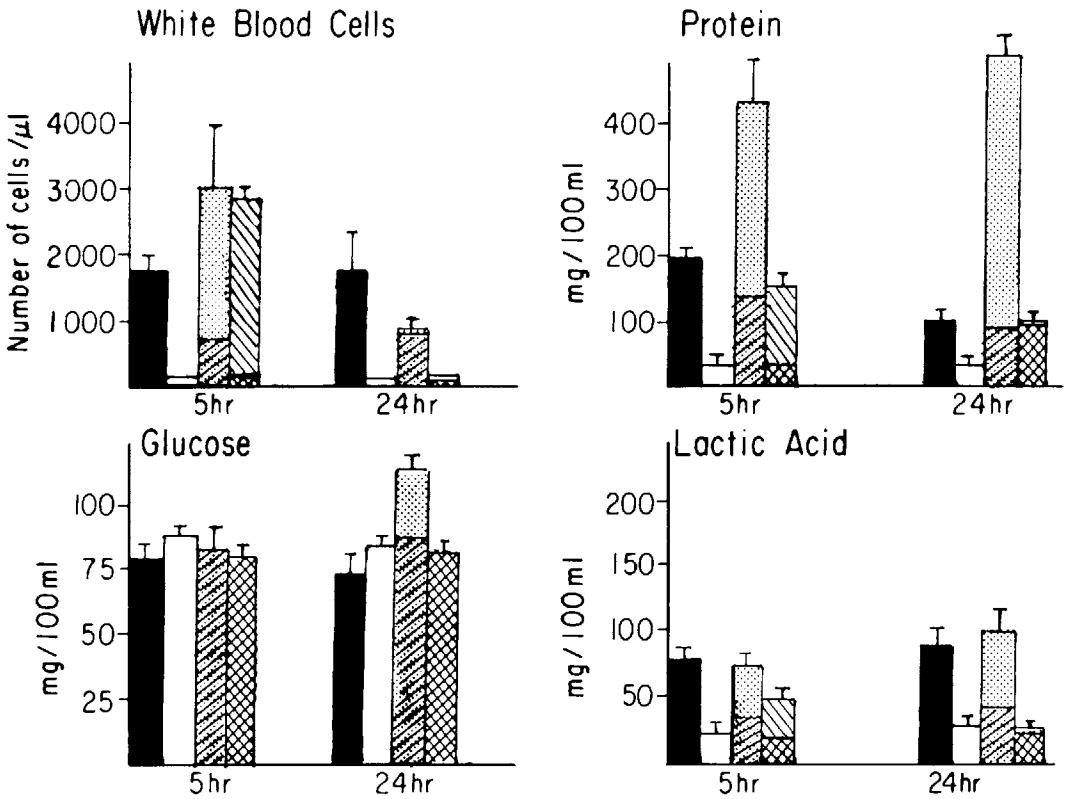

Figure 3. Changes in CSF cytochemical profile induced by various concentrations of killed pneumococci, purified cell wall, or capsular polysaccharide. Inocula of 2×10^7 heat-killed R_6 per 0.2 ml (■) or 2×10^7 heat-killed S_{III} per 0.2 ml (□) were each introduced into the cisterna magna of four rabbits. Inoculation of 0.2 ml of saline produced results identical to killed S_{III} ($P > .5$). Purified cell wall and capsular polysaccharide were each instilled into the cisterna magna of four rabbits at concentrations present on 10^6 cells (cell wall ▨, capsule □), or alternatively at 2 mg/0.2 ml (cell wall ▨, capsule □). CSF was withdrawn at 5 and 24 hr postinoculation and analyzed as indicated (mean ± SEM).

appeared in CSF at different times postinoculation; the striking finding was the correlation of the onset of the influx of leukocytes with the time when the bacterial density surpassed 10^5 organisms/ml. After the appearance of leukocytes, the CSF protein and lactic acid concentration began to increase. Both the sequence and magnitude of these changes were similar regardless of the presence or absence of capsule on the bacterium. Glucose concentration decreased only when organisms grew to a density $>10^6$ cfu/ml. Glucose concentrations decreased to <50 mg/dl during the inflammatory response to 10^8 unencapsulated organisms.

Difference in the CSF response induced by killed pneumococci. The lack of correlation between induction of the CSF inflammatory response and the presence of bacterial capsule suggested that bacterial components other than the capsule may be important in provoking the host response. The fact that all strains invoked a response at a similar bacterial density in CSF (i.e., 10^5) indicated that the inciting component had to be present in critical amounts to provoke a response. These two observations suggested that killed bacteria without capsule might also induce a CSF inflammatory response if given in the appropriate dose. Indeed, heat-killed preparations of unencapsulated bacteria when administered at $\geq 10^5$ cell equivalents per dose produced a CSF response remarkably similar to that of live bacteria (figure 3). Lower concentrations of killed organisms produced little inflammatory response in the CSF.

In contrast to heat-killed unencapsulated bacteria, heat-killed encapsulated inocula (10^6 and 10^8 cells) evoked only a minimal inflammatory response ($P < .01$, figure 3). This lack of activity did not appear to be due to alteration of the capsule by heat-

ing since heated-isolated capsular polysaccharide retained reactivity equal to that of untreated preparations (data not shown).

One striking difference in the CSF biochemical profile was noted when comparing live and killed inocula. CSF glucose concentrations remained normal during the response initiated by a killed inoculum (figure 3). On the other hand, both protein and lactic acid concentrations increased after the inoculation of live or killed unencapsulated bacteria. Inoculation of $>10^8$ killed bacterial cells failed to induce hypoglycorrhachia.

Comparison of isolated cell wall and capsular polysaccharide as inducers of CSF inflammatory response. Inoculation of $>10^5$ killed cells evoked an inflammatory response: this corresponds to $\sim$0.02 μg of cell wall material. Cell equivalents of capsular polysaccharide are difficult to calculate since the material is constantly shed into the medium during bacterial growth. However, 0.2 μg approximates the amount on 10^5 cells [9]. Figure 3 details the inflammatory response produced by inoculation of 10^6 cell equivalents of cell wall or capsular polysaccharide. Capsular polysaccharide produced little response at this concentration (mild increase in 24-hr CSF protein concentration). Cell wall material, on the other hand, evoked an inflammatory response similar to that of whole cells, including pleocytosis and an increase in protein and lactic acid concentrations; glucose concentration remained normal.

The inflammatory response generated by inocula of 2 mg of these two components is depicted in figure 3 ($\sim10^8$ cell equivalents of cell wall and $\sim10^9$ cell equivalents of capsular polysaccharide). At these concentrations both preparations induce inflammation although the magnitude of the response to cell wall is significantly greater than that to capsule.

Discussion

The CSF inflammatory response to all strains of pneumococci tested was similar, consisting of leukocytosis, an increase in protein and lactic acid concentrations, and hypoglycorrhachia. The fact that pneumococci with widely varying surface characteristics (differing capsular types or absence of capsule) could produce qualitatively and quantitatively similar CSF responses suggests that bacterial components other than capsule, yet common to all strains, are critical to the induction of CSF inflammatory changes. Potent inflammatory activity was found to reside in the cell wall of pneumococci. Most importantly, purified wall was active in inducing CSF inflammation at a concentration of $\sim10^6$ whole-cell equivalents. This number corresponds closely to the threshold at which CSF inflammation was found to begin with living bacteria (compare figures 1 and 2). In contrast, capsular polysaccharide was inactive at 2 μg per dose, an amount that represents not only $\sim10^6$ whole-cell equivalents but is also 10–100-fold greater than the concentration of capsule detected in rabbit CSF in meningitis [9].

Our experiments do not unequivocally identify the pneumococcal cell walls as the sole agents responsible for the inflammation observed in pneumococcal meningitis. A variety of bacterial components (intramembrane or intracellular) can induce inflammation in the CSF (authors' unpublished observations). Nevertheless, the pneumococcal cell wall is a plausible candidate for such a role, and our experiments clearly show that the specific activity of cell wall material as an inducer of inflammation is high enough to be operative at the bacterial concentrations observed in pneumococcal meningitis.

The mechanism whereby cell walls induce CSF inflammation is an yet unclear. The inflammatory activity of the cell wall has been noted in other experimental systems [10–14]. The pneumococcal cell wall is known to activate the alternative pathway of complement [15, 16] and could in this manner potentially generate chemotaxins and damage to the blood-brain barrier early in the course of infection. It should be noted, however, that CSF contains very little complement even during the later stages of meningitis [17, 18]. Chemotactic activity in CSF has been noted to be associated with heat stable substances of low molecular weight, some of which are generated during bacterial growth [19]. It is conceivable that cell wall components may be directly responsible for some of this chemotactic activity. Killed encapsulated strains were inactive, a result suggesting the presence of capsular material may mask the inflammatory activity of the underlying cell wall. It is conceivable that encapsulated bacteria produce inflammation by exposure of the underlying cell wall or by secretion of the cell wall material during growth.

Only actively multiplying organisms at $\geq10^6$ cfu/ml were found to induce hypoglycorrhachia. These results are similar to those obtained in vitro by Petersdorf et al. [20–22]. The mechanism of CSF hypoglycorrhachia in meningitis is unclear. The dis-

sociability of glucose and lactic acid changes would suggest that increases in the level of lactic acid cannot be taken as evidence of anaerobic glycolysis of decreasing CSF glucose. Our data suggest that the decrease in the level of glucose in CSF is caused by bacterial metabolic products other than the cell wall.

The potential clinical relevance of the CSF response to cell wall material rests on the correlation between the clinical outcome of meningitis and the degree of inflammation [23]. Pneumococcal cell wall components are released into the surrounding medium during stationary-phase autolysis and, perhaps more importantly, during treatment of pneumococci with β-lactam antibiotics. Thus, it is conceivable that bacterial death, when accompanied by lysis and release of cell wall, could aggravate CSF inflammation and affect the clinical outcome of disease deleteriously. This concept may explain in part the occurrence of deaths despite bacteriologic cure of the patient.

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